BARD Final Report IS-4899-16 R

Fibroblast growth factor signaling requirements for embryonic and placental development in ruminants

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Project award year: 2016
One year feasibility project

Final Report, BARD Research Project IS-4899-16 R
January 24, 2017

Title: Fibroblast growth factor signaling requirements for embryonic and placental development in ruminants

Submitters: Alan Ealy and Eran Gershon

As the committee will recall, a 1 year feasibility study was award ($100,000 for 1 year) to:

1. *Validate the efficiency and specificity of the shRNA or CRISPR for targeting each FGFR isoform, and*
2. *Examine the FGFRs receptors KO using shRNA or CRISPR on embryo livability and implantation in the cattle/sheep suggested models.*

Background

Embryonic and fetal losses in cattle and sheep occur, in large part, because of miscues in the complex array of physiologic, endocrine, paracrine, cellular, and molecular processes involved with embryonic and placental development and uterine function. The fibroblast growth factors (FGFs) family is known player in early pregnancy and embryo implantation. However, it remains unclear which activities are vital for pregnancy success.

Major conclusions, solutions and achievements

Dr. Ealy laboratory:

1) now has expertise with using CRISPR/Cas9 meganuclease technology to edit the genome of bovine embryos.
2) now can inject 50 to 60 zygotes each 1 hour, and at least 90% are successfully injected based on dye retention after several hours.
3) developed a guide RNA sequence (gRNA) targeting the FGFR2 transcriptional start site.
4) Proved that the percentage of cleaved embryos that achieved the blastocyst stage by day 8 post-in vitro fertilization was not different between non-injected zygotes, control-injected zygotes and FGFR2 gRNA-injected zygotes.
5) the targeting efficiency of genome alteration averaged 50% across the 5 replicates completed in this study. All modifications represented sequence deletions. Also, 85% of these edited genomes contained modifications in both alleles (i.e. biallelic editing), with 80% of them being heterozygous and 30% containing homozygous modifications. No work was completed to verify the absence of FGFR2 protein since all embryos were used for genotyping.

Dr. Gershon laboratory:

1) Has characterized the expression of FGFRs in ovine placenta at day 70 of pregnancy, demonstrating that FGFR2 is the most abundant FGF receptor in the placenta at this stage of pregnancy.
2) Designed and constructed two different shRNA sequences targeting FGFR2. Those sequences were designed to silence both ovine and bovine FGFR2.
3) Generated lentiviruses harboring those sequences. We currently verify the ability and efficiency of those two viruses to silence FGFR2 in ovine trophoblast cells.
3) Generated and established a method for trophoblast-specific lentiviral gene transfer in ovine.

Taken together, both laboratories are only a few studies away from having a gene editing procedure that can be used on a variety of targets in future years. In addition, we also acquired the ability to manipulate variety of genes expression (silencing or overexpress) specifically in ovine trophoblast cells and placenta.

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Thank you for your generous support of this 1-year feasibility project. I must concede that this study did not go as planned, but I do not consider it a failure. The funds were put to good use and several deliverables were achieved. A major positive outcome of the 2016 project was the creation of the Ealy-Gershon collaboration.

We have complimentary research skillsets, Gershon's lab benefit from the enormous knowledge of Dr. Ealy in embryology and his experience in sheep and cow hormonal treatment, embryo isolation and handling and different methods in embryo analysis, such histological examination and immunostaining. On the other hand, Ealy's lab consulted with Dr. Gershon regarding the different method to silence genes expression in the embryos and trophoblast cells.

These two laboratories share a common interest in embryology and we wish to continue to work together. We are grateful that BARD gave us the means to begin collaboration.

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Thank you for your generous support of this 1-year feasibility project. I must concede that this study did not go as planned, but I do not consider it a failure. The funds were put to good use and several deliverables were achieved. A major positive outcome of the 2016 project was the creation of the Ealy-Gershon collaboration. We have complimentary research skillsets, a common interest in embryology and we wish to continue to work together. We are grateful that BARD gave us the means to begin a collaboration.

Ealy Laboratory Report:

First, the laboratory now has expertise with using CRISPR/Cas9 meganuclease technology to edit the genome of bovine embryos. As will be discussed in greater detail below, the laboratory is only a few studies away from having a gene editing procedure that can be used on a variety of targets in future years.

The work completed by Dr. Ealy's laboratory in this 1-year feasibility study focused on the first request made by the committee, that being *to validate the efficacy of shRNA or CRISPR/Cas9 for targeting each FGFR isoform*. We have focused our efforts on working towards disrupting the FGFR2 gene. We focused on targeting the FGFR2. This receptor likely is the 'major player' in FGF actions in the embryo and developing placenta in mammals. Dr. Ealy's senior graduate student, Ms. Sarah McCoski, was placed onto this project. Ms. McCoski was tasked with 1) developing a functional microinjection system in the laboratory, and 2) establishing that the CRISPR/Cas9 technology could effectively modify the FGFR2 gene in bovine embryos. Ms. McCoski can now inject 50 to 60 zygotes each 1 hour, and at least 90% are successfully injected based on dye retention after several hours.

We also have evidence that the CRISPR/Cas9 technology will modify the FGFR2 gene. We developed a guide RNA sequence (gRNA) targeting the FGFR2 transcriptional start site. A study was completed to 1) determine the targeting efficiency of the FGFR2 gRNA, and 2) determine whether loss of FGFR2 function affects early embryo development. Treatment groups included 1) non-injected control zygotes, 2) zygotes microinjected only with Cas9 (injected controls), and 3) zygotes microinjected with FGFR2 gRNA and Cas9 (knockout group). Both the microinjection control group and FGFR2 gRNA microinjection group experienced a 10% reduction in cleavage rates versus the non-microinjected zygotes ($P<0.05$). This likely reflects a low rate of zygote death due to the microinjection procedure. The percentage of cleaved embryos that achieved the blastocyst stage by day 8 post-in vitro fertilization was not different between non-injected zygotes, control-injected zygotes and FGFR2 gRNA-injected zygotes (38 ± 6%, 34 ± 7% and 35 ± 3%, respectively). A majority of the morulae and blastocysts generated in the FGFR2 gRNA injected group were processed to examine the efficiency of FGFR2 gene editing. Sequence analysis determined that the targeting efficiency of genome alteration averaged 50% across the 5 replicates completed in this study (range 41 to 63% among replicates). No insertions were detected. All modifications represented sequence deletions. The magnitude of targeted deletion ranged from 5 to 103 base pairs. All deletions spanned the transcriptional start site target. Also, 85% of these edited genomes contained modifications in both alleles (i.e. biallelic editing), with 80% of them being heterozygous and 30% containing homozygous modifications. No work was completed to verify the absence of FGFR2 protein since all embryos were used for genotyping. These observations clearly indicate that CRISPR/Cas9 technology can effectively target FGFR2.

Gershon Laboratory Report:

First we characterized the expression of the Different FGFRs in ovine placenta. We detected all four FGFRs receptor at the mRNA level. We also found that FGFR1 and 2 are expressed in the ovine placenta at day 70 at the protein level. Our results also revealed that FGFR2 mRNA is expressed 20,000 fold higher than the mRNA of FGFR1, 3 and 4. An elevated expression of FGFR2 was also detected at the protein level as compared to FGFR1 protein expression. Based on these results we have decided to focus on targeting FGFR2 in the ovine trophoblast cells.

We have identified two potential shRNA sequences to silence FGFR2 in ovine. The criteria for the selected shRNA sequences were sequences that can silence both ovine and bovine FGFR2, for future experiments. We have cloned these sequences into the lentiviral plasmid and generated the

viruses harboring those two sequences. We also infected ovine blastocysts with either shControl virus or with those two viruses. All embryos infected with either the control virus or FGFR2 viruses survived and exhibit normal and healthy morphology. Currently, we verify the absence of FGFR2 protein in the infected embryos.

We have also developed a novel lenti-viral based method for gene manipulation specifically in ovine trophoblast cells. Embryos donor ewes were treated with superovulation protocol and intra-uterine inseminated using sperm collected from rams. The conceptuses were flushed at day 8, blastocyst stage. During the embryo flushing procedure, we also counted the number corpora lutea to asses the rate of insemination and flushing success. In two experiments conducted so far, we have inseminated 9 sheep. We have succeeded to flush embryos from 6 sheep (between 4-15 embryos from a sheep, the flushing rate was between 60-90% as determined by the corpora lutea count). Embryos were incubated with control lentiviruses expressing green fluorescent protein (GFP) under the regulation of either eif1a or cytomegalia virus (CMV) promoter. Then, embryos infected with eif1a promoter were transferred to 4 ewes and embryos infected with CMV promoter viruses were transferred to 5 ewes (3 embryos per sheep). As detected at day 35 of pregnancy by ultrasound method, 2 sheep retrieved with eif1a virus and 3 sheep retrieved with CMV virus were pregnant.

At day 70 of pregnancy, pregnant sheep were sacrificed and the placentomes, uteri and embryo were monitored for GFP expression. Only the placentome (the embryonic side of the placenta), expressed GFP. No GFP signal was detected in either the embryos or uteri. GFP was detected in all placentomes isolated from all sheep used in this experiment. In addition, infection with both different promoters resulted in GFP expression specifically in the placentomes.

To summarize, Based on Dr. Ealy previous findings, we predict that loss of FGFR2 will not adversely affect embryo development before the blastocyst stage. With that said, we wish to further refine the system before we pursue the functional aspects of embryo development any further.

To summarize the findings, all indications are that the CRISPR/Cas9 and shRNA interference are effective at targeting FGFR2 gene mutations. In addition, all indications are also that we have developed a method for trophoblast-specific lentiviral gene transfer in ovine. Those technologies combined will be a great resource for the laboratories for continued investigations into the actions of FGFs and other embryotrophic factors.

Publications for Project IS-4899-16 R

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun

APPENDIX

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Gershon Laboratory Report:

We wished to characterize the role of FGFRs in embryo implantation. Specifically: 1) Define the specific FGFRs that control early embryonic development in bovine embryos. 2) Define the specific FGFRs that control placental development, implantation and hormone production in ovine embryos.

1. Define the specific FGFRs expressed in ovine placenta

RNA and protein were extracted from placentomes isolated from sheep at day 70 of pregnancy. Using PCR analysis with specific primers to the different FGF receptors, we found that all four FGF receptors are expressed in the ovine placentomes. Quantitive PCR analysis using the specific primers, revealed that FGFR2 mRNA expression is 20000 fold higher as compared to the other receptor. We further examined the FGFR1 and FGFR2 protein levels using specific antibody to each receptor by western blot technique. We found that in correlation with the mRNA levels, FGFR2 protein expression was higher as compared to FGFR1 expression.

Finally, we have sectioned some of the placentomes isolated and preformed immunofluorescence staining with the FGFR1 and FGFR2 antibodies. We found that both receptors are expressed in the villi of the embryo placentomes. The staining of FGFR2 exhibit a stronger signal than the FGFR1 staining, supporting the results obtained by the quntitive PCR and western blot analysis. Taken together, the results from all three experiments suggest that FGFR2 is the most abundant FGF receptor in the ovine placenta.

2. Generating shRNA FGFR2 vector

Next, We have identified and designed two potential shRNA sequences to silence FGFR2 in ovine. The criteria for the selected shRNA sequences were sequences that can silence both ovine and bovine FGFR2, for future experiments conduct in both species.

We have cloned the sequences into lentiviral internal ribosome entry site vector, p156RRLSINpptCMVGFPPRE, that incorporates cytomegalovirus-driven GFP as a marker. The cloning itself took more times than anticipated due to priblems in reaching sufficient amount from the plasmid, sequencing that did not work well and screening many colonies to achieve the plasmid harboring the shRNA sequence in the right orientation. Once the plasmid was generated and the correct orientation of the sequence was validated, we have generated the viral particles harboring FGFR2-targeted shRNA cassettes using HEK293T cells.

We then infected ovine blactocysts with either shFGFR2 virus or control virus, and monitored their survival for 8 hours. All embryos from all groups survived and exhibit normal morphology. Those embryos were taken for further analyses of verify the silencing of FGFR2 protein in the infected embryos, which is currently being preformed.

3. Developing a novel lenti-viral based method for gene manipulation specifically in ovine trophoblast cells.

We have developed a new method for gene silencing or overexpression specifically in the trophoblast cells using lenti-viral based system. Embryos donor ewes were treated with superovulation protocol. Then, we have tried to breed donor ewes with genetically related rams (three-quarter siblings) and flush the conceptuses on day 8, blastocyst stage. However, the hormonal treatment used at the superovulation protocol interferes with the sperm movement and no embryos were achieved.

We therefore, ewes were treated with superovulation protocol and using the laparoscopic procedure, we intra-uterine injected fresh sperm collected from rams. Then, at day 8, embryos were washed from uteri of the embryo donor ewes. During the embryo flushing procedure, we also counted the number corpora lutea to asses the rate of insemination and flushing success.

In two experiments conducted, we have inseminated 9 sheep. We have succeeded to flush embryos from 6 sheep. In the first experiment we retrieved embryos from 2 ewe, and in the second experiment from 4 ewes. between 4-15 embryos were flushed from a sheep. The flushing rate was between 60-90% as calculated by number of embryos collected devided by the number of corpora lutea counted. Embryos were split to two groups: 1) embryos incubated with control lentiviruses that incorporates cytomegalovirus (CMV)-driven GFP as a marker or 2) embryos incubated with control lentiviruses that incorporates eif1a-driven GFP as a marker. We have used two different promoters to examine which promoter is activate in the ovine cells. After 4-6 hours of incubation, embryos were washed from the viruses and transferred to recipient ewes. Between 3-4 embryos were implanted in each recipient ewe. 4 ewes were retrieved with embryos infected with viruses incorporates eif1a-driven GFP and 5 ewes were retrieved with embryos incorporates CMV-driven GFP.

At day 35 of pregnancy, ultrasound examination was preformed to detect whether the recipient sheep got pregnant. Two of the recipient ewes retrieved with eif1a-driven GFP (1 in each experiment conducted), and 3 sheep retrieved with CMV-driven GFP virus were pregnant (1 ewe in the first experiment and 2 ewes in the second experiment).

In the first experiment, both ewes was sacrificed at day 70 0f pregnancy and the placentomes, uteri and embryo were lighted with blue led lamp immediately upon their removal from the ewe for detection of GFP expression. Only the placentome (the embryonic side of the placenta), expressed GFP. No GFP signal was detected in either the embryos or uteri. The expression of GFP in the embryonic side of the placentome was further verified by preforming immunostaining with GFP specific antibody on sections prepared from the isolated placentomes. As a negative control, we used sectioned incubated with no primary antibody to GFP.

In the second experiment, ending these days, one pregnant sheep was sacrificed at day 50 of pregnancy, and the placentomes, uteri and embryo were lighted with blue led lamp immediately upon their removal from the ewe for detection of GFP expression. Only the placentome (the embryonic side of the placenta), expressed GFP. No GFP signal was detected in either the embryos or uteri. The expression of GFP in the embryonic side of the placentome will be further verified by preforming immunostaining with GFP specific antibody on sections that will be prepared from the isolated placentomes. As a negative control, we will used sectioned incubated with no primary antibody to GFP.

The rest of the ewes from the present experiment, will also be sacrefised at day 70, and the same analysis and procedures will be used to verify the specific expression of GFP only in the embryonic side of the placentomes. In addition, in this experiment a pregnant ewe with non-infected embryos will be scarefised and the placentomes, embryo and uteri will serve as a negative control to GFP expression.

In summary, all indications are that we have developed a method for trophoblast-specific lentiviral gene transfer in ovine. This technology will be a great resource for the laboratories for continued investigations into the actions of FGFs and other embryotrophic factors.

Ealy Laboratory Report:

We have focused our efforts on working towards disrupting the FGFR2 gene. Specifically, 1) developing a functional microinjection system in the laboratory, and 2) establishing that the CRISPR/Cas9 technology could effectively modify the FGFR2 gene in bovine embryos.

1. Developing a functional microinjection system.

This system has been developed. We had to overcome an instrument problem and now are using a new system (purchased using other funds) comprised of a SMZ1500 Nikon Stereomicroscope with Narshige pneumatic injection and holding injectors. Ms. McCoski can now inject 50 to 60 zygotes each 1 hour, and at least 90% are successfully injected based on dye retention after several hours.

2. Establishing that CRISPR/Cas9 technology could effectively modify the FGFR2 gene.

We have evidence that the CRISPR/Cas9 technology will modify the FGFR2 gene. This work was completed with the assistance of Dr. Kiho Lee, an Assistant Professor in Animal & Poultry Sciences at Virginia Tech. Dr. Lee studies gene-editing technologies in porcine embryos. The logic with this target site was that interrupting this region of the gene will interfere with FGFR2 transcription.

A study was completed to 1) determine the targeting efficiency of the FGFR2 gRNA, and 2) determine whether loss of FGFR2 function affects early embryo development. Treatment groups included 1) non-injected control zygotes, 2) zygotes microinjected only with Cas9 (injected controls), and 3) zygotes microinjected with FGFR2 gRNA and Cas9 (knockout group). The injections occurred 16-18 h after fertilization.

A majority of the morulae and blastocysts generated in the FGFR2 gRNA injected group were processed to examine the efficiency of FGFR2 gene editing. DNA was extracted from individual embryos and PCR was used to amplify the target region (450 bp amplification). A portion of the PCR product was electrophoresed to examine whether product size was affected by the gene editing. The reminder of the PCR product was used Sanger sequencing (Virginia Tech DNA Sequencing Core Facility).

Sequence analysis determined that the targeting efficiency of genome alteration averaged 50% across the 5 replicates completed in this study (range 41 to 63% among replicates).

The efficiency at which this is accomplished is well-within the range where further experimentation can be completed. The absence of adverse embryonic development effects in the FGFR2-edited embryos is not a huge pause for concern. Until very recently, Dr. Lee's group was using a similar approach for their various studies in pig embryos, where one gRNA targeting the transcriptional start site was used. Targeting efficiency was great in that work (usually very high, 80+%), however, he found several instances where targeted genes still transcribed functional proteins. We propose this was caused by alternative

transcriptional start site utilization. Because of this, we think it is prudent to modify our approach before proceeding with the actual functional studies.

Our plan is to generate several gRNAs that target different regions of the FGFR2 gene. We are in the process of identifying these regions. We will continue to use the original gRNA and are examining sites within the FGF binding domain and the first kinase region as second and third gRNA targets. Also, we will need to verify knockout success by examining the loss of FGFR2 protein (via immunofluorescence) using an antibody reacting with the C-terminal region of FGFR2 and with signaling interference testing (e.g. ERK1/2 responses to FGF2 treatment).